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Calcium-induced Cardiac Mitochondrial Dysfunction Is Predominantly Mediated by Cyclosporine A-dependent Mitochondrial Permeability Transition Pore

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Background and Aims. Cardiac mitochondrial Ca^{2+} overload plays a critical role in mechanical and electrical dysfunction leading to cardiac cell death and fatal arrhythmia. Because Ca^{2+} overload is related to mitochondrial permeability transition, reactive oxygen species (ROS) production and membrane potential ($\Delta\Psi m$) dissipation, we probed the mechanistic association between Ca^{2+} overload, oxidative stress, mitochondrial permeability transition pore (mPTP) and mitochondrial calcium uniporter (MCU) in isolated cardiac mitochondria.

Methods. Various concentrations of Ca^{2+} (5–200 μM) were used to induce mitochondrial dysfunction. Cyclosporin A (CsA, an mPTP blocker) and Ru360 (an MCU blocker) were used to test its protective effects on Ca^{2+}-induced mitochondrial dysfunction.

Results. High concentrations of Ca^{2+} (≥100 μM) caused overt mitochondrial swelling and $\Delta\Psi m$ collapse. However, only slight increases in ROS production were detected. Blocking the MCU by Ru360 is less effective in protecting mitochondrial dysfunction.

Conclusions. A dominant cause of Ca^{2+}-induced cardiac mitochondrial dysfunction was mediated through the mPTP rather than MCU. Therefore, CsA could be more effective than Ru360 in preventing Ca^{2+}-induced cardiac mitochondrial dysfunction. © 2012 IMSS. Published by Elsevier Inc.

Key Words: Cardiac mitochondria, Calcium, Permeability transition, Reactive oxygen species, Membrane potential.

Introduction

Intracellular Ca^{2+} overload of cardiomyocytes can lead to both electrical and mechanical dysfunction (1). Mitochondria play a crucial role in buffering cytosolic Ca^{2+} for maintaining physiological Ca^{2+} signals (2). When mitochondrial Ca^{2+} uptake exceeds a certain threshold level, the mitochondria can no longer regulate the intramitochondrial Ca^{2+}, resulting in the opening of mitochondrial permeability transition pore (mPTP) (3,4). Opening of mPTP allows the influx of water and solutes (<1500 Da) into the matrix causing mitochondrial swelling, mitochondrial membrane potential ($\Delta\Psi m$) collapse, and eventually cell death (2). However, the mechanism by which Ca^{2+} mediates the mPTP opening in cardiac cells is still controversial and varies in different models (5). Ca^{2+} overload also leads to the increase in reactive oxygen species (ROS) production (6–8). However, the relationship between Ca^{2+} overload, mPTP opening, $\Delta\Psi m$ change and oxidative stress is still elusive.

Cyclosporin A (CsA) is regarded as a potent inhibitor of mPTP by preventing the interaction of cyclophilin D and adenine nucleotide translocator (ANT). It has long been used to identify mPTP activity in various tissues (9,10) and has been proven to be a cardioprotective agent (11). Therefore, in this study we investigated the mechanistic association between Ca^{2+} overload, mitochondrial

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dysfunction, oxidative stress and the mPTP opening in cardiac mitochondria. Moreover, because the mitochondrial calcium uniporter (MCU) has been proposed as a principal portal for Ca^{2+} influx (12–14), we also investigated the effect of Ru360, a selective MCU blocker, against mitochondrial dysfunction. In the present study we tested the hypotheses that the mechanism of Ca^{2+}-induced cardiac mitochondrial dysfunction is mainly via the mPTP and the MCU.

Materials and Methods

Ethics Approval

This study was approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University.

Animals

Male Wistar rats (n = 18, 350–400 g) were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. All animals were housed in a room with controlled temperature maintaining between 22 and 25°C with a constant 12 h light/dark cycle. Rats were fed with standard rat pellet diet and water ad libitum.

Experimental Protocols

Isolated cardiac mitochondria were used in all experiments. Various doses of $CaCl_2$ (5, 10, 100, 200 μM) were applied to isolated cardiac mitochondria for 10 min before the measurement of mitochondrial swelling, ROS production and ΔΨm dissipation. In addition, the effect of the mPTP blocker (CsA) and the MCU blocker (Ru360) on attenuating Ca^{2+}-induced mitochondrial dysfunction was also investigated. Isolated cardiac mitochondria were pretreated with CsA at the concentration of 5 μM for 30 min before the application of $CaCl_2$ (15) or with Ru360 10 μM for 5 min (16).

Isolation of Cardiac Mitochondria

Cardiac mitochondria were isolated according to the method described previously (17,18). Male Wistar rats weighing 350–400 g were anesthetized by isoflurane and thiopental (80 mg/kg), and the hearts were perfused with ice-cold normal saline. The heart was then removed, minced with razors, and homogenized in ice-cold isolation buffer containing sucrose 300 mM, TES 5 mM and EGTA 0.2 mM, pH 7.2 (4°C). The homogenate was centrifuged at 800 g for 5 min. The supernatant was collected and centrifuged at 8800 g for 5 min. The mitochondrial pellet was resuspended in ice-cold isolation buffer and finally centrifuged at 8800 g for 5 min. The mitochondrial pellet was suspended in 2 ml of respiration buffer containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES, and 5 mM KH_2PO_4, pH 7.4, at 37°C. Mitochondrial protein concentration was measured using bicinchoninic acid (BCA) assay (19).

Identification of Cardiac Mitochondria with Electron Microscopy

Electron microscopy was used to identify the morphology of cardiac mitochondria (20). Isolated mitochondria were fixed overnight by mixing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C. The pellet was then rinsed in cacodylate buffer and postfixed in 1% cacodylate-buffered osmium tetroxide for 2 h at room temperature. Next, the mitochondrial pellet was dehydrated in a graded series of ethanol and embeded in Epon-Araldite. The pellet was then cut into ultrathin sections (60–80 nm thick) using a diamond knife, placed on copper grids and stained with uranyl acetate and lead citrate. Finally, mitochondria were observed with a transmission electron microscope.

Measurement of Cardiac Mitochondrial Swelling

Mitochondria suspension was added with respiration buffer to a final concentration of 0.4 mg/ml. Cardiac mitochondrial swelling was determined by the decrease of light absorbance at 540 nm using a microplate reader (18–21).

Measurement of ROS Production

Cardiac mitochondria (0.4 mg/mL) were incubated with 2 μM dichlorohydro-fluorescein diacetate (DCFDA) at 25°C for 20 min. In the presence of ROS, DCFDA was oxidized to DCF and the fluorescence increased. Fluorescence was determined at 485 nm for excitation and 530 for emission. The ROS level was expressed in arbitrary units of fluorescence intensity of DCF (18).

Measurement of Mitochondrial Membrane Potential Change (ΔΨm)

The mitochondrial membrane potential change of isolated cardiac mitochondria were evaluated by using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (22,23). JC-1 is a lipophilic, cationic dye which can pass into mitochondria. When mitochondria have high ΔΨm, JC-1 is in the aggregate form, which shows red fluorescence. With low ΔΨm (i.e., depolarized state), it remains in monomeric form showing green fluorescence. The isolated mitochondria (0.4 mg/mL) were incubated with 310 nM JC-1 at 37°C for 30 min (18). Fluorescence intensity was measured using a fluorescent microplate reader. The green fluorescence of JC-1 monomer was excited at 485 nm and the emission was noticed at 530 nm. The red fluorescence of J-aggregates was excited at 485 nm and the emission was detected at 590